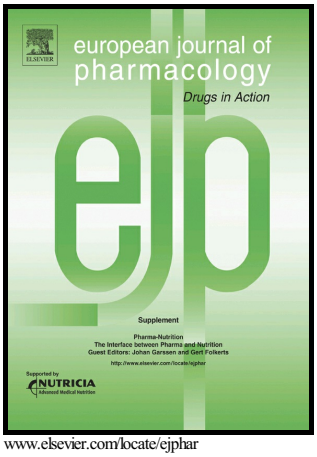

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Positive allosteric modulation of native and recombinant $GABA_A$ receptors by hops prenylflavonoids

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Abstract

Hops are a major component of beer that is added during brewing. In addition to its wide range of bioactivity, it exhibits neuroactive properties as a sedative and sleeping aid. The compounds responsible for this activity are yet to be revealed and understood in terms of their pharmacological properties. Here we evaluated the potential of several hops flavonoids in modulating the GABAergic activity and assessed their selectivity to $GABA_A$ receptors subtypes. GABA-potentiating effects were measured using [^{3}H]ethynylbicycloorthobenzoate (EBOB) radioligand binding assay in native and recombinant α1β3γ2, α2β3γ2 and α6β3δ receptors expressed in HEK293 cells. Flumazenil sensitivity of GABA-potentiating effects and [^{3}H]Ro 15-4513 binding assay were used to examine the flavonoids binding to benzodiazepine site. The prenylflavonoids xanthohumol (XN), isoxanthohumol (IXN) and 8-prenylnaringenin (8PN) potentiated GABA-induced displacement of [^{3}H]EBOB binding in a concentration-dependent manner. The IC_{50} for this potentiation in native $GABA_A$ receptors were 29.7 μM, 11.6 μM, 7.3 μM, respectively. In recombinant receptors, the sensitivity to prenylflavonoid potentiation of GABA-induced displacement of [^{3}H]EBOB binding followed the order α6β3δ > α2β3γ2 > α1β3γ2 with the strongest inhibition observed by 8PN in α6β3δ (IC_{50} = 3.6 μM). Flumazenil had no significant effect on the prenylflavonoid-induced displacement of [^{3}H]EBOB binding and [^{3}H]Ro 15-4513 displacement from native $GABA_A$ receptors was only detected at high micromolar concentrations (100 μM). We identified potent prenylflavonoids in hops that positively modulate GABA-induced responses in native and αβγ/δ recombinant $GABA_A$ receptors at low micromolar concentrations. These GABAergic modulatory effects were not mediated via the high-affinity benzodiazepine binding site.

Keywords

$GABA_A$ receptors, Allosteric modulation, radioligand binding*, Humulus lupulus*, Flavonoids, Pharmacodynamics

1. Introduction

Hops (*Humulus lupulus* L., Cannabaceae) are essential components of beer that is added during the brewing process. The female inflorescences of this plant contain resins, essential oils, and polyphenols that preserve beer and bring its distinctive aroma and bitterness. In addition, it exhibits neuroactive properties that made it potentially useful in traditional medicine as a mild sedative and sleeping aid (Zanoli and Zavatti, 2008). These effects have been suggested to be mediated by an increase in the function of γ-aminobutyric acid type A ($GABA_A$) receptors, the major sites of fast synaptic inhibition in the central nervous system (CNS) (Sieghart, 1995), e.g. positive modulation of GABA-induced responses at $GABA_A$ receptors has been observed earlier by extracts of beer and hops (Aoshima et al., 2006; Sahin et al., 2016).

$GABA_A$ receptors are members of the Cys-loop superfamily of ligand-gated ion channels (Sigel and Steinmann, 2012). They are pentameric protein complexes consisting of various subunits. A total of 19 mammalian genes coding for $GABA_A$ receptor subunits belonging to 8 subunit classes have been cloned: α1-α6, β1-β3, γ1-γ3, δ, ε, π, θ and ρ1-ρ3 (Olsen and Sieghart, 2008). Most of the receptor subtypes consist of α, β and γ subunits in a subunit stoichiometry of 2α:2β:1γ (McKernan and Whiting 1996; Tretter et al., 1997). The receptor pentamer contains an intrinsic chloride channel that is opened by the binding of GABA. Upon activation, the influx of chloride ions into the neuron hyperpolarizes and/or stabilizes the membrane potential and thus inhibits the neuron (Olsen and Sieghart, 2008). $GABA_A$ receptors are positively modulated by several clinically used important drugs, such as benzodiazepines, barbiturates, steroids, and anaesthetics (Sieghart, 1995). The γ subunit is necessary for benzodiazepine binding and γ2 is the isoform that exists in > 90% of αβγ receptors (McKernan and Whiting 1996; Pritchett et al., 1989; Tretter et al., 1997). In native $GABA_A$ receptors, γ2 can be found in combination with all α subunit variants, the subunit class that defines benzodiazepine pharmacology (Pritchett et al., 1989).

To better understand the neuropharmacological properties of hops, its potentially neuroactive compounds should be identified and their mechanisms of action to be revealed at the receptor level. Xanthohumol, the main prenylflavonoid in hops, and its metabolites isoxanthohumol and 8-prenylnaringenin have received much attention in the recent years due to their wide range of bioactivity against microbes, oxidative stress, inflammation and cancer (Botta et al., 2005; Karabín et al., 2016; Di Viesti et al., 2011; Yang et al., 2015; Zanoli et al., 2005; Zanoli and Zavatti, 2008). These phytocompounds deserve further evaluation for their potential benefits in insomnia and anxiety management as it is now evident that several naturally occurring flavonoids modulate $GABA_A$ receptors at flumazenil-sensitive and -insensitive sites (Hanrahan et al., 2011; Johnston, 2015).

In the present study, we investigated GABAergic modulatory effects and receptor subtype selectivity of hops flavonoids using radioligand binding assays in native and recombinant $GABA_A$ receptors. We further assessed if the active hops compounds exert their modulatory effect via $GABA_A$ receptor-associated benzodiazepine binding site. Radioligands used were [^{3}H]ethynylbicycloorthobenzoate (EBOB), a potent noncompetitive blocker of $GABA_A$ receptor (Huang and Casida, 1996) and [^{3}H]Ro 15-4513, a partial inverse agonist of $GABA_A$ receptor benzodiazepine binding site (Corda et al., 1989; Sieghart et al., 1987).

2. Materials and methods

2.1. Reagents

The radioligands [propyl-2,3-^{3}H]EBOB (specific activity 48 Ci/mmol), and [7,9-^{3}H]Ro 15-4513 (28 Ci/mmol) were purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA, USA). GABA and picrotoxin were from Sigma Chemical Co. (St. Louis, MO, USA). Flumazenil (Ro 15–1788) was kindly provided by F. Hoffmann-La Roche Ltd (Basel,

Switzerland). The hops flavonoids catechin, quercetin, quercitrin, and rutin (Gerhäuser, 2009) were purchased from Chengdu Biopurify Phytochemicals (Chengdu, Sichuan, China).

2.2. Synthetic Chemistry

As a raw material for isolation of xanthohumol, by-products obtained after extraction of hops with supercritical CO_2 in industrial installations located in the New Chemical Syntheses Institute, in Puławy, Poland were used. Isolation of xanthohumol and chemical cyclization of xanthohumol into isoxanthohumol by base-catalyzed isomerization was performed as described earlier (Anioł et al., 2008). 8-prenylnaringenin was obtained by demethylation of isoxanthohumol following the method described by Stompor et al., (2017). Spectroscopic data of the isolated hop compounds were in agreement with literature values (Stompor et. al., 2013; Stompor et al., 2017; Potaniec et. al., 2014). The purity of obtained compounds was above 98% according to high performance liquid chromatography (HPLC).

2.3. Animals

A total of 30 native male Sprague-Dawley rats (age: 3-4 months) were obtained from Central Animal Laboratory of the University of Turku (Turku, Finland). The rats were euthanized by decapitation; their forebrains along with midbrain were dissected, frozen on dry ice and stored at -70 ^{o}C. All procedures were in compliance with protocols approved by the Institutional Animal Care and Use Committee of the University of Turku.

2.4. Preparation of brain membranes

Rat fore/midbrain membrane preparation was modified from Squires and Saederup method (2000) and essentially performed as described in Uusi-Oukari et al. (2014). Rat forebrain along with midbrain region was homogenized into 10 mM Tris-HCl, pH 8.5 buffer containing 2 mM ethylenediaminetetraacetic acid (EDTA) using an Ultra-Turrax T25 (Janke & Kunkel IKA labortechnik) for 20 s at 9500 rpm. The homogenates were centrifuged at 20 000 *g* for 10 min at +4 ^{o}C and the resulting pellets were washed 3 times by resuspension and re-centrifugation with 10 mM Tris-HCl, pH 8.5 buffer containing 0.2 M NaCl and 5 mM

EDTA. The resulting pellets were then suspended in ice-cold H_2O and centrifuged. The pellets were again washed 3 times with Tris, pH 8.5/NaCl/EDTA as described above and the pellets finally suspended in 10 mM Tris-HCl, pH 7.4 and frozen at -70 ^{o}C.

2.5. Cell culture and recombinant $GABA_A$ receptor expression

Human embryonic kidney 293 (HEK293) cells were grown in culture medium containing DMEM powder (Dulbecco's modified Eagle's medium, Gibco, Gaithersburg, MD) and 3.7 g $NaHCO_3$ in each 1 liter of Milli-Q water supplied by 10% fetal bovine serum (Gibco, Gaithersburg, MD; USA), 50 000 U/l penicillin and 50 mg/l streptomycin (Sigma-Aldrich, St Louis, MO, USA). The cultured cells were maintained in incubation at 37°C under 95% humidity and 5% CO_2. For transfection the cells were detached with a hypertonic solution (40 mM Tris-HCl, pH 7.4, 1 mM EDTA, 250 mM NaCl) and divided in 1:3 ratio to reach 50% confluency after 24 h. Transfection was then performed using Ca_2PO_4 precipitation method (Graham and van der Eb, 1973). Rat cDNAs (α1, α2, α6, β3, γ2S, δ) in pRK5 plasmids (Uusi-Oukari et al., 2000) under the control of cytomegalovirus (CMV) promoter were used for transfections. The plasmids coding for the different receptor subunits were used in 1:1:1 ratio for transfections containing $GABA_A$ receptor subtypes α1β3γ2, α2β3γ2, and α6β3δ. For each 100 mm culture plate, a transfection mixture was prepared to contain 5 µg of each subunit plasmid, 450 µl of sterile milli-Q water, 50 µl 2.5 M $CaCl_2$ solution (J. Baker, Austin, TX, USA) and 500 µl 2x HBS buffer (pH 7.00). The cells were harvested 48 h after transfection using a detaching buffer (10 mM Tris-HCl, 0.15 M NaCl, 2 mM EDTA) and centrifuged at 20 000 *g* for 10 min at +4 ^{o}C. The resulting pellets were suspended in 10 mM Tris-HCl, pH 7.4, containing 0.15 M NaCl, then stored at -20 ^{o}C for 24 h before conducting binding assays.

2.6. [^{3}H]EBOB binding assay

[^{3}H]EBOB binding assay was performed in principle as described by Uusi-Oukari and Maksay (2006). Frozen membranes were thawed, washed once by centrifugation at 10,000

g for 10 min and resuspension in assay buffer (50 mM Tris-HCl, 120 mM NaCl, pH 7.4), and resuspended finally in the same buffer. Triplicate and quadruplicate samples of rat brain or HEK293 cell membranes, respectively, were incubated at room temperature with shaking for 2 h in 50 mM Tris-HCl, pH 7.4, containing 120 mM NaCl with 1 nM or 2 nM [^{3}H]EBOB in the absence or presence of 2 µM GABA and different concentrations of hops compounds (10 nM - 30 µM) in a total volume of 400 µl. Non-specific binding was determined in the presence of 100 µM picrotoxin. To separate bound and unbound radioligand, the incubation was terminated by filtration of the samples with a Brandel Cell Harvester (model M-24, Gaithersburg, MD, USA) onto Whatman GF/B glass fiber filters (Whatman International Ltd., Maidstone, UK). The samples were rinsed three times with 5 ml of ice-cold 10 mM Tris-HCl, pH 7.4. Air-dried filters were immersed in 3 ml of Optiphase HiSafe 3 scintillation fluid (Perkin Elmer Life and Analytical Sciences, Boston, MA, USA) and radioactivity determined in Hidex 600 SL liquid scintillation counter (Hidex, Turku, Finland). Non-specific binding was subtracted from total binding to obtain the specific binding values.

2.7. [^{3}H]Ro 15-4513 binding assay

Rat brain membranes were prepared and [^{3}H]Ro 15-4513 binding assays performed in principle as described in Uusi-Oukari and Korpi (1990). Quadruplicate samples were incubated on ice at 4 ^{o}C with shaking for 1 h in assay buffer (see above) with 2 nM [^{3}H]Ro 15-4513 in the absence and presence of prenylflavonoids (10 and 100 µM) in a total volume of 300 µl. Non-specific binding was determined in the presence of 10 µM flumazenil. The incubation was terminated as described for [^{3}H]EBOB binding.

2.8. Protein measurement

The protein concentrations of membranes for all radioligand binding studies were determined with the Bio-Rad Coomassie blue dye-based protein assay kit (Hercules, CA, USA) according to manufacturer's instructions.

2.9. Statistics

GraphPad Prism 7 software (GraphPad, San Diego, CA, USA) was used in statistical testing and curve fitting of the radioligand displacement data. Inhibition data were fit to the sigmoidal dose-response equation with a variable Hill Slope:

$Y=Bottom + (Top-Bottom)/(1 + ((X^{HillSlope})/(IC_{50}^{HillSlope})))$

where Y is the percentage of control binding, Bottom = 0 when non-specific binding is subtracted from all binding values, Top is the maximum value, and X is the test compound concentration. Nonlinear least squares regression analysis was used for estimation of IC_{50} values which are presented as means ± S.E.M. The statistical comparisons were performed with unpaired *t*-test and one-way ANOVA followed by Tukey's or Dunetts *post hoc* test. P-values of less than 0.05 were considered significant.

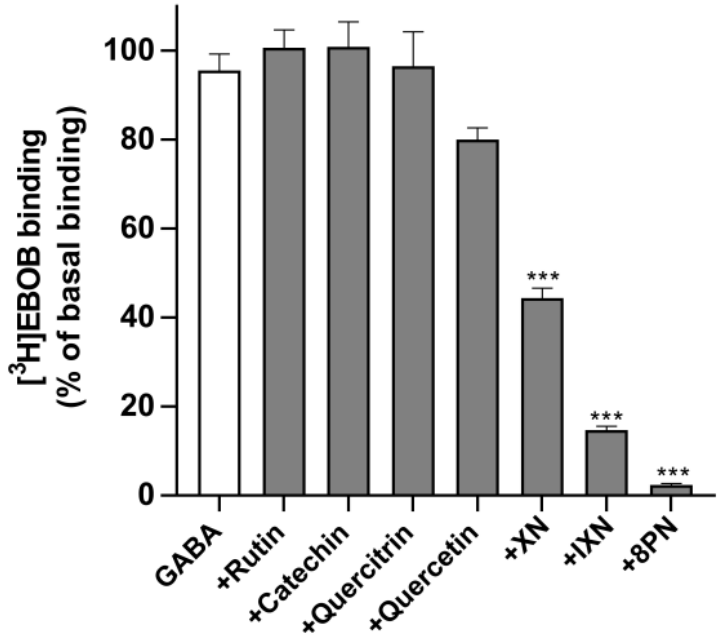
3. Results

3.1. Allosteric potentiation of GABA-induced [^{3}H]EBOB displacement

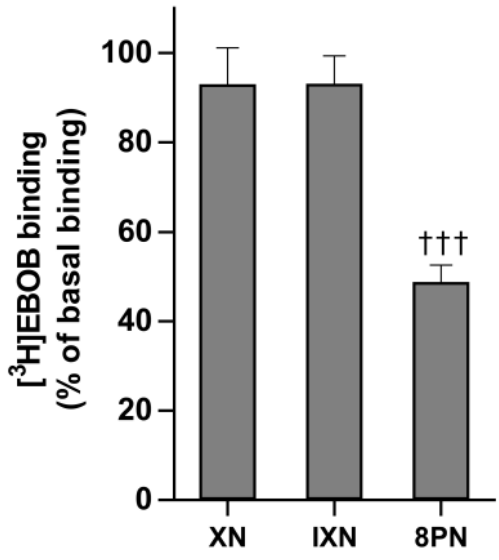
The displacement of [^{3}H]EBOB binding from rat forebrain membranes was studied first with 30 µM concentrations of hops flavonoids in the presence of 2 µM GABA. This concentration of 8-prenylnaringenin, isoxanthohumol and xanthohumol displaced 97.6 ± 0.3%, 85.2 ± 0.8%, and 55.6 ± 2.3% of specific [^{3}H]EBOB binding, respectively ($P < 0.001$, one-way ANOVA, Dunett's *post hoc* test). On the other hand, the flavonoids rutin, catechin, quercitrin, and quercetin showed no significant effects on [^{3}H]EBOB binding (Fig. 1A) and hence were not further assessed. In the absence of added GABA to adequately washed membranes, the displacing effect was only evident with 8-prenylnaringenin (30 µM) as it displaced 51.3 ± 3.9% of specific [^{3}H]EBOB binding ($P < 0.001$, one-way ANOVA, Dunett's *post hoc* test) (Fig. 1B).

Figure 1

A



B.



Concentration series for displacement of [^{3}H]EBOB binding was further conducted for the three active prenylflavonoids in the presence of 2 µM GABA. Each of these compounds showed a concentration-dependent inhibition of [^{3}H]EBOB binding in rat forebrain

membranes (Fig. 2). GABA alone at 2 µM displaced 10.2 ± 1.6% of 1 nM [^{3}H]EBOB basal binding ($P < 0.01$). 8-prenylnaringenin and isoxanthohumol at 3 µM concentration inhibited specific [^{3}H]EBOB binding in the presence of GABA by 26.6 ± 2.8% ($P < 0.001$) and 19.9 ± 2.5% ($P < 0.001$), respectively. However, this concentration for xanthohumol had no significant effects on [^{3}H]EBOB displacement by GABA. The IC_{50} values of the three compounds for rat forebrain membranes were in low micromolar range (Table 1) where isoxanthohumol and 8-prenylnaringenin had 2.5 and 4.1-fold higher inhibition potency compared to xanthohumol, respectively ($P < 0.001$, one-way ANOVA followed by Tukey's *post hoc* test).

Figure 2

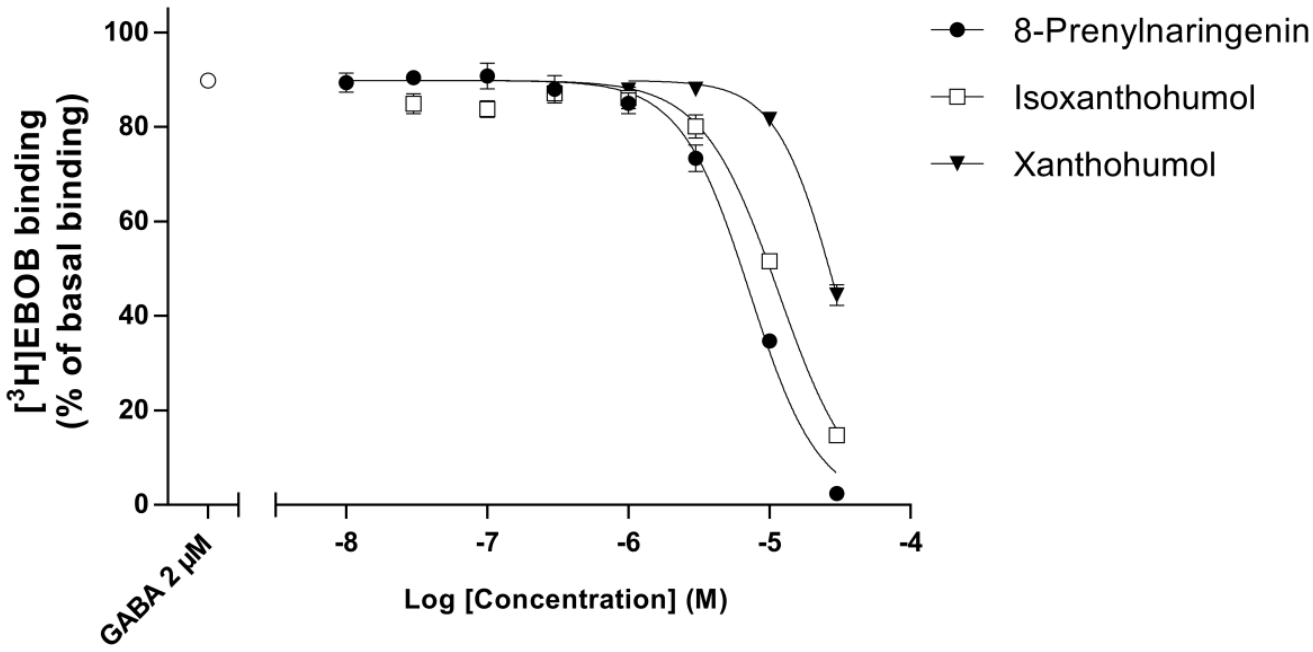


Table 1 IC_{50} values for prenylflavonoid potentiation of GABA-induced [^{3}H]EBOB displacement in native $GABA_A$ receptors. The values represent mean ± S.E.M. (n = 3). [a]P < 0.001, significantly different from the corresponding value for xanthohumol (one-way ANOVA followed by Tukey's *post hoc* test)

Compound	IC_{50}
Xanthohumol	29.7 ± 0.8 µM
Isoxanthohumol	11.6 ± 0.7 µM[a]
8-Prenylnaringenin	7.3 ± 0.4 µM[a]

The concentration curves for displacement of [^{3}H]EBOB binding by prenylflavonoids from three recombinant $GABA_A$ receptor subtypes are presented in Fig. 3, and the IC_{50} values are summarized in Table 2. All tested compounds potentiated GABA-induced displacement of [^{3}H]EBOB binding in a concentration-dependent manner. The sensitivity of recombinant receptor subtypes followed the rank order α6β3δ > α2β3γ2 > α1β3γ2. Isoxanthohumol and 8-prenylnaringenin showed 2.6 fold higher inhibition potency in α6β3δ compared to α1β3γ2 receptor subtype, respectively. The highest inhibition potency observed was with 8-prenylnaringenin in α6β3δ combination (IC_{50} = 3.6 ± 0.5 µM, n=4). However, the effect was absent with xanthohumol at the lowest concentration tested (3 µM) in α1β3γ2.

Figure 3

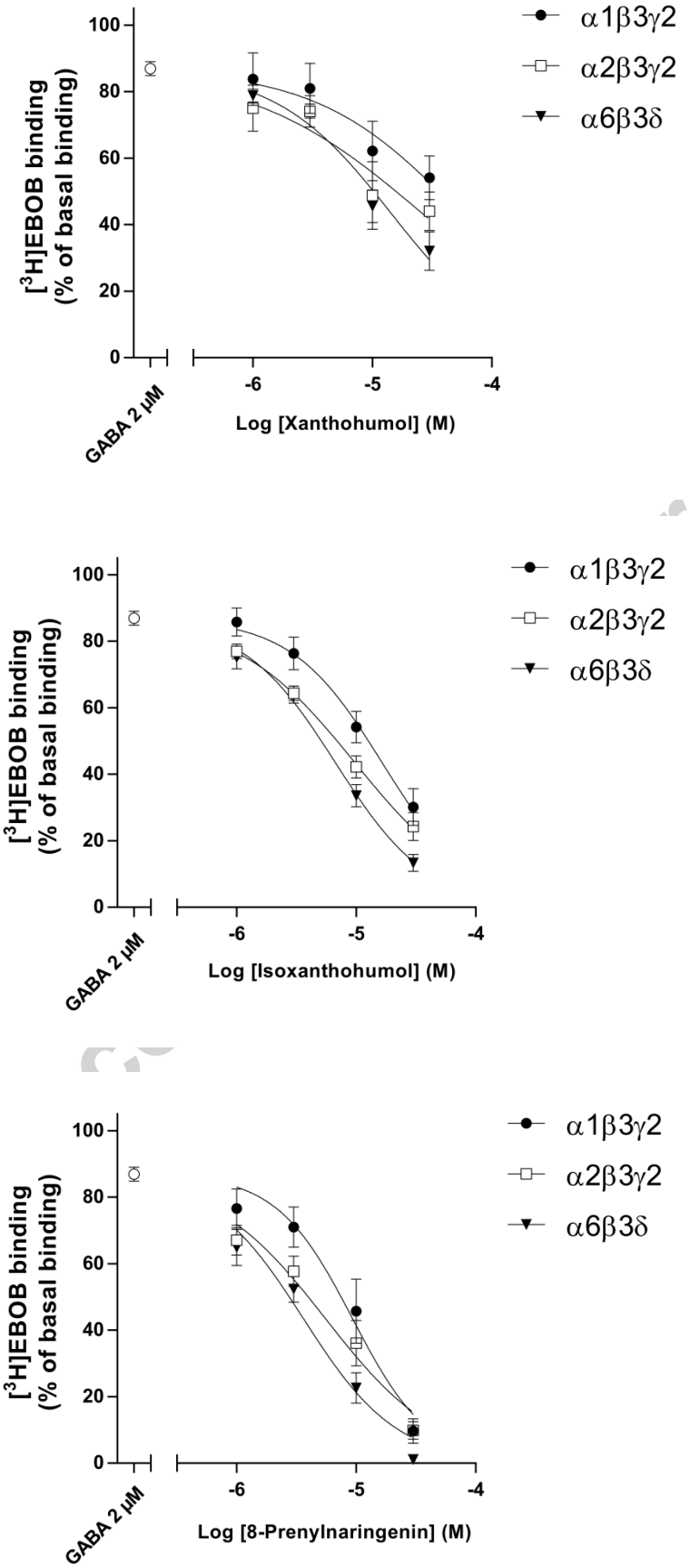


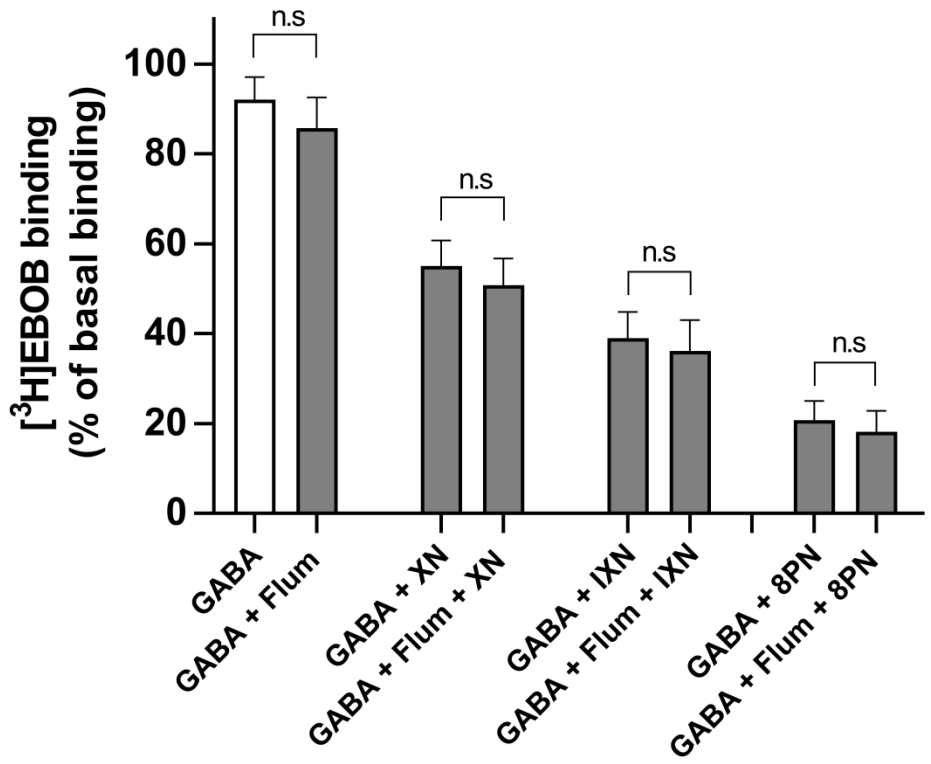
Table 2. IC_{50} values of prenylflavonoid potentiation of GABA-induced [^{3}H]EBOB displacement in recombinant receptors expressed in HEK293 cells. The values represent mean ± S.E.M. (n = 4).

	α1β3γ2	**α2β3γ2**	**α6β3δ**
Xanthohumol	54 ± 31 μM	26 ± 11 μM	14 ± 2.1 μM
Isoxanthohumol	16.5 ± 2.3 μM	9.8 ± 1 μM	6.7± 0.6 μM
8-Prenylnaringenin	9.3 ± 1.6 μM	5.5 ± 0.9 μM	3.6 ± 0.5 μM

3.2. Allosteric modulation of $GABA_A$ receptor at benzodiazepine binding site

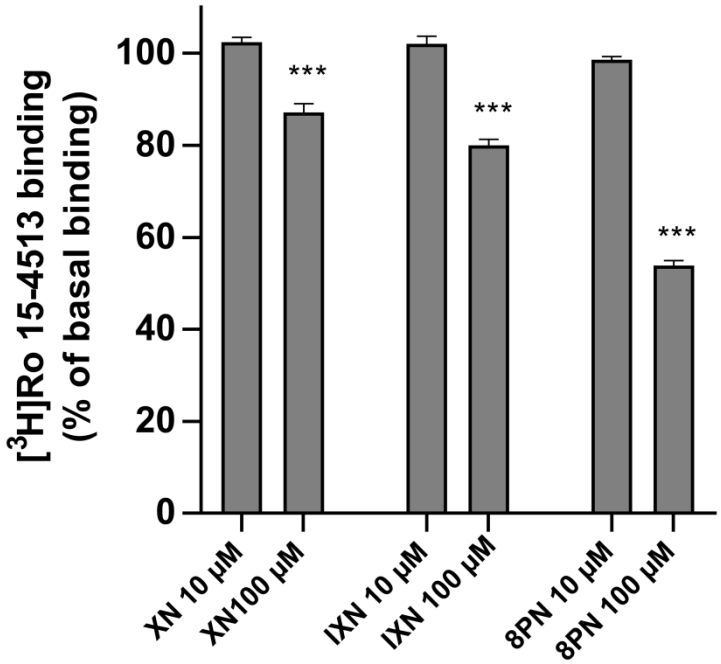
We examined the ability of flumazenil to reverse prenylflavonoid potentiation of GABA-induced [^{3}H]EBOB displacement through its antagonizing effect on $GABA_A$ receptor-associated benzodiazepine site. Prenylflavonoid potentiation of GABA-induced [^{3}H]EBOB displacement was not affected by the addition of flumazenil as there was no significant difference between flumazenil treated and non-treated samples (Fig. 4, $P > 0.05$).

Figure 4



To assess further whether the beer flavonoids bind to flumazenil-sensitive $GABA_A$ receptor benzodiazepine site, the displacement of [^{3}H]Ro 15-4513 binding by the flavonoids in rat forebrain membranes was studied (Fig. 5). The results indicated a total absence of displacement at 10 µM for all candidate compounds. The displacement was only detected at a relatively high concentration (100 µM) where 8-prenylnaringenin, isoxanthohumol, and xanthohumol displaced 46.1%, 20.0%, and 12.9% of specific [^{3}H]Ro 15-4513 binding, respectively ($P < 0.001$, unpaired t-test).

Figure 5

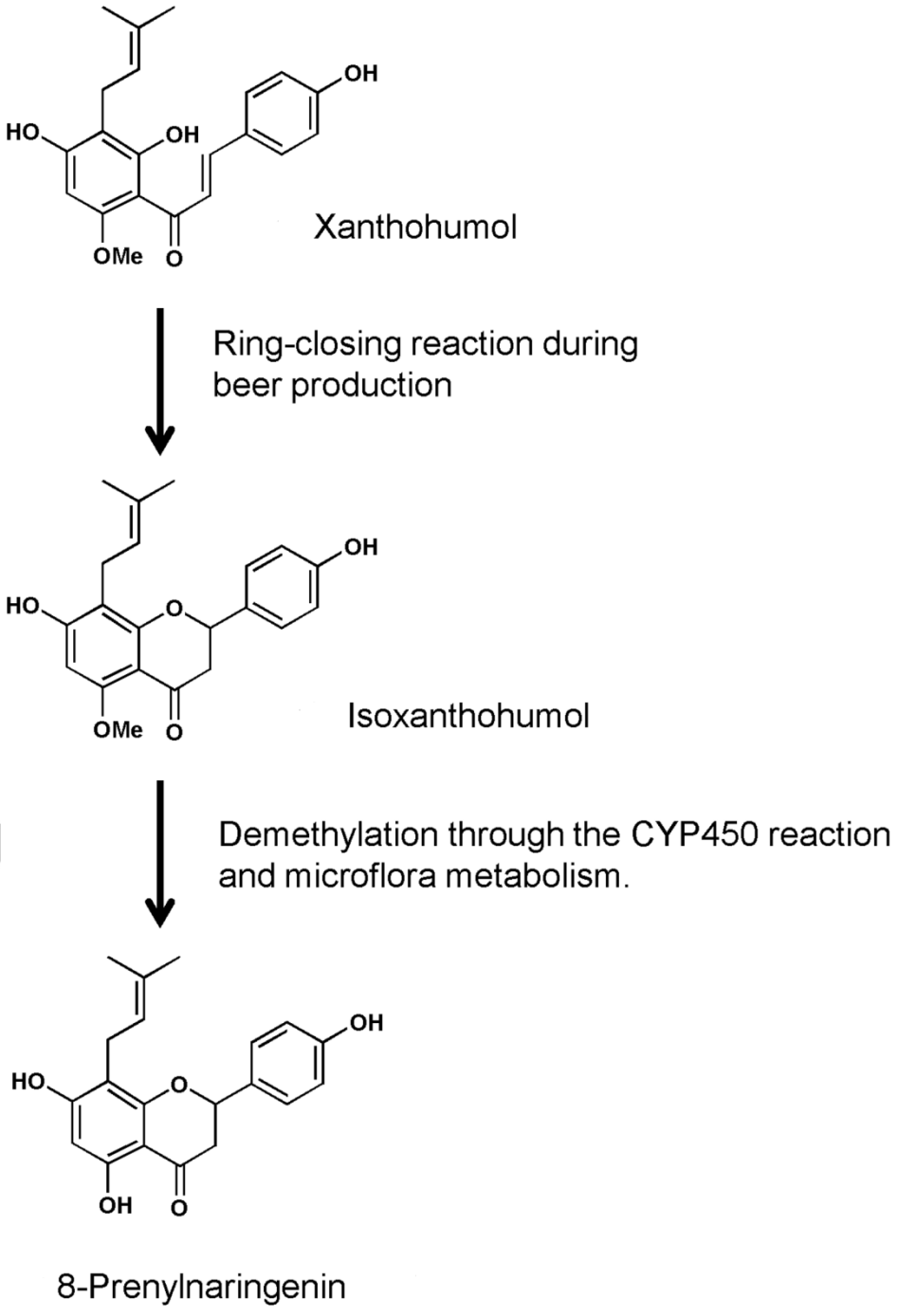


4. Discussion

In the present study, we identified three structurally related prenylflavonoids present in hops that positively modulate GABA-induced responses in native and αβγ/δ recombinant $GABA_A$ receptors at low micromolar concentrations. However, their site of action is flumazenil-insensitive and not likely to be the classical benzodiazepine site in $GABA_A$ receptors. The sensitivity of $GABA_A$ receptors to prenylflavonoid potentiation of GABA-induced displacement of [^{3}H]EBOB binding followed the order: 8-prenylnaringenin > isoxanthohumol > xanthohumol. Despite being the major component of hop hard resins, xanthohumol is usually present in beer with trace amounts ranging from 0.002 to 1.2 mg/l (Stevens et al., 1999). This is attributed to its loss in the boiling step during brewing where xanthohumol is cyclized to isoxanthohumol, the most common flavonoid present in beer

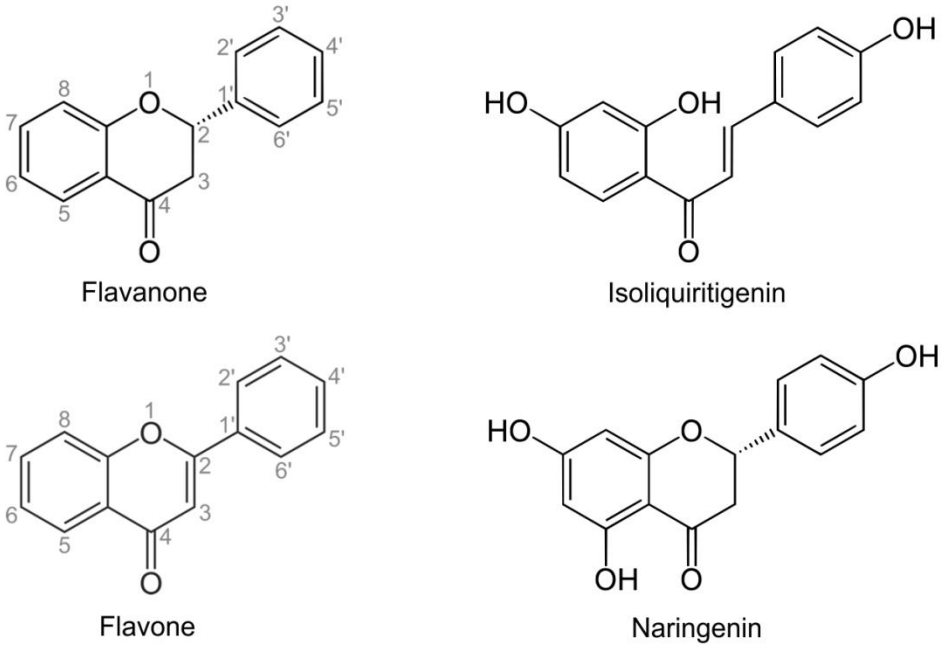
(range: 0.04-3.44 mg/l) (Stevens et al., 1999). Therefore brewers add xanthohumol-enriched extracts to increase its amounts in beer (Karabín et al., 2013; Magalhães et al. 2012; Wunderlich et al., 2005). Upon beer consumption, isoxanthohumol undergoes biotransformation to 8-prenylnaringenin (Possemiers et al., 2005; 2006), the most potent phytoestrogen identified so far (Kitaoka et al., 1998; Milligan et al., 1999) (Fig. 6).

Figure 6



Our findings are in accordance with a fluorescence correlation spectroscopy study that showed xanthohumol modulatory effects on muscimol binding to $GABA_A$ receptors (Meissner and Häberlein, 2006). More recently, in rat hippocampus, xanthohumol has been shown to inhibit evoked glutamate release via its activity on $GABA_A$ receptor (Chang et al., 2016). Structurally similar compounds such as isoliquiritigenin, a natural flavonoid found in liquorice root that shares the same basic chalcone structure with xanthohumol (Fig. 7), is proven to positively modulate $GABA_A$ receptors showing hypnotic and anxiolytic properties in mice (Cho et al. 2011; Jamal et al. 2008). Naringenin, a natural flavonoid that is found in tomatoes and citrus fruits has similar flavanone moiety as 8-prenylnaringenin with the exception that it lacks a prenyl group at its C-8 position (Fig. 7). Radioligand binding studies showed that (S)-naringenin extracted from *Mentha aquatic* displaces [^{3}H]flumazenil in cerebral cortex rat membranes. However, the activity was very low in contrast to classical benzodiazepines (e.g. diazepam) as the IC_{50} value was 2.6 mM (Jäger et al., 2007). Interestingly, 8-prenylnaringenin displaced 46% of [^{3}H]Ro 15-4513 binding at 100 µM and was able to displace 51% of [^{3}H]EBOB binding at 30 µM even in the absence of GABA. The latter result can be attributed to ability of 8-prenylnaringenin to directly activate $GABA_A$ receptors in a similar manner as barbiturates at high concentration (Sieghart, 1995).

Figure 7



4.1. *$GABA_A$ receptor subtype selectivity of hops prenylflavonoids and related compounds*

Based on our results, the modulatory effects of hops prenylflavonoids are not dependent on the presence of γ or δ subunit suggesting a major role of α/β interfaces in $GABA_A$ receptor pentameric complex. Nevertheless, the subtype selectivity of these compounds is obvious since relatively higher potency is observed in α6β3δ recombinant receptors. For example, the IC_{50} values of isoxanthohumol and 8-prenylnaringenin in α6β3δ were 2.6 fold lower than those obtained for α1β3γ2 recombinant receptors. In native receptors, δ subunit exists extrasynaptically and only in α6/4β combinations as a major α6β2/3δ subtype in cerebellar granule cells and as α4β2/3δ subtype in thalamus, cerebral cortex, hippocampal dentate gyrus granule cells, caudate-putamen and in the nucleus accumbens (Jechlinger et al., 1998; Pirker et al., 2000; Pöltl et al., 2003). These receptors are benzodiazepine insensitive with high GABA affinity and they mediatine tonic inhibition in

several types of neurons in the brain (Saxena & Macdonald, 1996; Semyanov et al., 2003; Mortensen & Smart, 2006; Stórustovu and Ebert, 2006). The potentiation of GABA-induced response in αβδ receptors is found to be highly sensitive to ethanol already at low millimolar concentrations that produce mild intoxicating effects in humans (Hanchar et al., 2005; Sundstrom-Poromaa et al., 2002; Wallner et al., 2003). This suggests that prenylflavonoids capable of positively modulating these receptors may also potentiate the intoxicating effects of ethanol, i.e. strongly hopped beer would produce stronger intoxication than non-hopped beer with equivalent amount of ethanol.

Our study revealed that the selectivity of allosteric modulation is also dependent on α subunit as it was evident that the tested prenylflavonoids were more efficacious in α2β3γ2 than α1β3γ2 recombinant receptors. These findings are consistent with Hall et al. (2014) results on 6-methoxyflavanone, a synthetic flavonoid that shares the same flavanone moiety with isoxanthohumol and 8-prenylnaringenin. 6-methylflavanone has been shown to positively modulate $GABA_A$ receptors in a flumazenil-insensitive manner with higher efficacy on α2β2γ2L than α1β2γ2L receptors. 6-methylflavanone was also found to cross the blood-brain barrier (BBB) and exhibit *in vivo* anxiolytic effects in mice (Akbar et al., 2017). It was demonstrated in earlier studies that α1 subunit-containing $GABA_A$ receptors mediate sedation while α2 subunit-containing $GABA_A$ receptors mediate anxiolysis (Löw et al., 2000; McKernan et al., 2000; Rudolph et al, 1999). Hence, assessing the selectivity to α subunit in $GABA_A$ receptors is essential when developing selective anxiolytic drugs based on hops prenylflavonoids with the least possible sedative effects.

According to our results, catechin at 30 μM concentration showed no modulatory activity on $GABA_A$ receptors. This is consistent with Campbell et al. (2004) study indicating that (+)-catechin (1-100 μM) has no effect on GABA-induced responses in α1β2γ2L recombinant receptors. However, a direct agonist activity was later observed with (+)-catechin in α4β3δ recombinant receptors at a high micromolar concentration (100 μM)

although it had no effect on [^{3}H]muscimol and [^{3}H]flunitrazepam binding to $GABA_A$ receptors (Eghorn et al., 2014). The effect of rutin on [^{3}H]EBOB binding was also absent in our study. Rutin has been found to induce anxiolytic-like effects in rats (Hernandez-Leon et al., 2017). However, these findings suggested that benzodiazepine-sensitive site is not likely to be involved in rutin activity as the effects were not antagonized by flumazenil and only partially blocked with picrotoxin. There are discrepancies in the results concerning quercetin that needs to be further assessed. Although quercetin and its glycoside analogue (quercitrin) had no effect on [^{3}H]EBOB binding in our study, an earlier electrophysiology study showed negative modulatory effects by quercetin on α1β1γ2S recombinant receptors at the same 30 μM concentration (Goutman et al., 2003). On the other hand, quercetin was able to induce anxiolytic-like effects in mice that were antagonized by TACA (trans-4-aminocrotonic acid), a potent agonist at ρ-containing $GABA_A$ receptors (Borowicz et al., 2005; Jung and Lee 2014). Quercitrin has also been found to induce anxiolytic-like effects; however, these were shown to be mediated by serotonin 5-HT(1A) receptors and not $GABA_A$ receptor benzodiazepine binding site (Li et al., 2016).

4.2. Considerations on the role benzodiazepine binding site in allosteric modulation

Despite the fact that hops prenylflavonoids enhanced GABA-induced responses in receptors sensitive to benzodiazepines (α2β3γ2 and α1β3γ2), this enhancement was insensitive to the antagonizing effects of flumazenil on the high-affinity benzodiazepine site. Moreover, the binding of [^{3}H]Ro 15-4513 is insensitive to the tested prenylflavonoids in native $GABA_A$ receptors at 10 μM. This radioligand displacement is only detected at high micromolar concentrations (100 μM), while the GABA-potentiating effect of the prenylflavonoids was already seen at the low micromolar range in [^{3}H]EBOB assay. Hence, our results suggest that the GABAergic modulatory effects of hops prenylflavonoids are not mediated via the high-affinity benzodiazepine binding site. The planar structure of flavonoids is a determinant for binding to $GABA_A$ receptors at benzodiazepine site (Dekermendjian et al., 1999). For example, flavanones lack a double bond between C-2 and C-3 as compared

to flavones which make it less planar (Fig. 7). Due to planarity, several natural and synthetic flavones bind to benzodiazepine sites with high affinity (Medina et al., 1998).

4.3. Flavonoids permeability across the blood-brain barrier

A key property that determines the potential of neuroactive flavonoids to modulate receptor function in the CNS is the ability to cross the BBB. Despite the lack of BBB penetration experiments on hops prenylflavonoids, results obtained from previous *in vivo* studies supports their crossing. Xanthohumol has been found to improve cognitive function in mice and was detected in the cerebral cortex and hippocampus upon five days of intraperitoneal administration (40 mg/kg) (Zamzow et al., 2014). However, the concentration detected in the brain was only 4 nM, significantly lower than its active range on $GABA_A$ receptors. Till date there are no published studies on the actual concentrations found in the brain for isoxanthohumol and 8-prenylnaringenin upon beer drinking or direct administration. This needs to be further investigated in order to correlate their pharmacological activity with the physiological brain-mediated effects. Rad et al. (2006) demonstrated that the concentration of luteinizing hormone (LH) in the brain was decreased in postmenopausal women upon the administration of 8-prenylnaringenin (750 mg). LH is secreted by the anterior pituitary gland and regulated by the gonadotropin-releasing hormone (GnRH) that is synthesized and released from the hypothalamus. This process can be regulated by estrogen-like compounds such as 8-prenylnaringenin suggesting its ability to cross the BBB through to its interaction with hypothalamo–pituitary axis (Christoffel et al., 2006). There is clear evidence from *in vitro* and *in vivo* studies that naringenin crosses the BBB conforming to its high lipophilicity (Youdim et al., 2004). Hence, the addition of the prenyl group to naringenin as in the case of 8-prenylnaringenin could further lead to higher lipophilicity and in turn enhanced BBB penetration (Botta et al., 2005).

5. Conclusions

To conclude, our study provides a proof of concept that hops prenylflavonoids act as potent positive modulators in native and recombinant $GABA_A$ receptors at low micromolar concentrations. The highest potency is observed in 8-prenylnaringenin with a relatively higher subtype selectivity to δ-containing $GABA_A$ receptors. This modulation suggests a potentiation of the intoxicating effects of ethanol in beer which needs further investigation with functional and behavioural studies. Moreover, the identification of neuropharmacologically important compounds of hops and their beneficial effects could enable their use as lead molecules in the development of selective and safe drugs for anxiety, insomnia and alcohol withdrawal.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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Figure captions:

Figure 1 Displacement of [^{3}H]EBOB (1 nM) binding in rat forebrain membranes by different hops compounds (30 µM) in the presence (A) and absence (B) of GABA (2 µM). The values represent means ± S.E.M., n= 3, measured in triplicates. ***P < 0.001 for the significance of difference from [^{3}H]EBOB basal binding in the presence of GABA alone; †††P < 0.001 for the significance of difference from [^{3}H]EBOB basal binding without GABA (one-way ANOVA followed by Dunnett's *post hoc* test).

Figure 2 Displacement curves of [^{3}H]EBOB (1 nM) binding in rat forebrain membranes by 4-8 concentrations of test compounds in the presence of GABA. The values represent means ± S.E.M., n= 3, measured in triplicates. IC_{50} values were calculated from the inhibition curves by nonlinear least squares regression.

Figure 3 Displacement curves of [^{3}H]EBOB (2 nM) binding in recombinant $GABA_A$ receptors expressed in HEK293 cells with four concentrations of xanthohumol (A), isoxanthohumol (B) and 8-prenylnaringenin (C) in the presence of GABA (2 µM). The values represent means ± S.E.M., n= 4, measured in quadruplicates. IC_{50} values were calculated from the inhibition curves by nonlinear least squares regression.

Figure 4 Inhibition of [^{3}H]EBOB (1 nM) binding in rat forebrain membranes by xanthohumol (30 µM), isoxanthohumol (10 µM) and 8-prenylnaringenin (10 µM) in the presence of GABA (2 µM) and flumazenil (2 µM). The values represent means ± S.E.M., n= 3, measured in triplicates; n.s, non-significant difference from the corresponding [^{3}H]EBOB basal binding (unpaired t-test).

Figure 5 Inhibition of [^{3}H]Ro 15-4513 (2 nM) binding in rat forebrain membranes by 10 µM and 100 µM concentrations of xanthohumol, isoxanthohumol, and 8-prenylnaringenin. The values represent means ± S.E.M., n= 3, measured in quadruplicate. ***P < 0.001, significantly different from [^{3}H]Ro 15-4513 basal binding (one-way ANOVA followed by Dunnett's *post hoc* test).

Figure 6 Transformation of xanthohumol to 8-prenylnaringenin (modified from Mukai et al., 2012)

Figure 7 Chemical structures of flavanone, flavone, isoliquiritigenin and naringenin

Credit Roles

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